RESEARCH

Effects of SGLT2 inhibition on lipid transport in adipose tissue in type 2 diabetes

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Abstract

SGLT2 inhibition induces an insulin-independent reduction in plasma glucose causing increased lipolysis and subsequent lipid oxidation by energy-consuming tissues. However, it is unknown whether SGLT2 inhibition also affects lipid storage in adipose tissue. Therefore, we aimed to determine the effects of SGLT2 inhibition on lipid storage and lipolysis in adipose tissue. We performed a randomized, double-blinded, placebo-controlled crossover design of 4 weeks of empagliflozin 25 mg and placebo once-daily in 13 individuals with type 2 diabetes treated with metformin. Adipose tissue fatty acid uptake, lipolysis rate and clearance were measured by ^{11}C-palmitate PET/CT. Adipose tissue glucose uptake was measured by ^{18}F-FDG PET/CT. Protein and gene expression of pathways involved in lipid storage and lipolysis were measured in biopsies of abdominal s.c. adipose tissue. Subjects were weight stable, which allowed us to quantify the weight loss-independent effects of SGLT2 inhibition. We found that SGLT2 inhibition did not affect free fatty acids (FFA) uptake in abdominal s.c. adipose tissue but increased FFA uptake in visceral adipose tissue by 27% ($P < 0.05$). In addition, SGLT2 inhibition reduced GLUT4 protein ($P = 0.03$) and mRNA content ($P = 0.01$) in abdominal s.c. adipose tissue but without affecting glucose uptake. In addition, SGLT2 inhibition decreased the expression of genes involved in insulin signaling in adipose tissue. We conclude that SGLT2 inhibition reduces GLUT4 gene and protein expression in abdominal s.c. adipose tissue, which could indicate a rebalancing of substrate utilization away from glucose oxidation and lipid storage capacity through reduced glycerol formation.

Key Words

- SGLT2 inhibitors
- lipolysis
- lipogenesis
- lipid uptake

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Introduction

Sodium-glucose cotransporter 2 (SGLT2) inhibitors are effective antidiabetic agents with remarkable cardiovascular benefits (1). Even though SGLT2 inhibitors primarily exert their pharmacological effect in the kidneys, the insulin-independent lowering of plasma glucose has several pleiotropic consequences that may contribute to overall cardioprotection (2). This includes a 2–2.5 kg weight loss and a significant impact on adipose tissue with an increase in lipid mobilization and lipolysis (3, 4). However, SGLT2 inhibitor-related weight loss is substantially less pronounced than would be expected from the number of calories lost through the increased glucose excretion which is ascribed to a compensatory increase in food intake (5, 6). Therefore, SGLT2 inhibitors may not only affect whole-body lipolysis but also regional lipid storage. However, this has not yet been investigated.

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Adipose tissue is an energy reservoir that stores fatty acids as triglyceride during times of caloric excess and releases fatty acids for oxidation and ketogenesis between meals and during fasting (7). The balance between storage and release of fatty acids is regulated by several hormones and other metabolic factors that are affected by SGLT2 inhibition. SGLT2 inhibition reduces insulin concentration, a potent inhibitor of lipolysis (8), and therefore causes increased lipolysis. As a counterbalancing mechanism, increased free fatty acids (FFA) levels result in increased circulating ketone bodies which inhibit lipolysis through metabolite-sensing G-coupled receptors on adipocytes (3, 9). In addition to the effects on lipolysis, reduced insulin concentrations could also affect lipid storage through its regulating effects on lipoprotein lipase (LPL) (10). LPL hydrolyses triglycerides from circulating lipoproteins, whereby fatty acids are generated and subsequently taken up by adipocytes through a combination of fatty acid transport proteins and passive membrane transport (11, 12). Adipose tissue does also takes up glucose, primarily through insulin-stimulated GLUT4 uptake (13, 14). Glucose is not only a substrate for oxidation but also importantly converted into glycerol which is necessary for re-esterification of fatty acids into triglycerides (15). Hence, SGLT2 inhibition possibly has the dual potential of increasing lipolysis and reducing adipose tissue lipid storage.

To elucidate the effects of SGLT2 inhibition on the balance between storage and release of fatty acids in adipose tissue, we investigated the effects of 4 weeks of SGLT2 inhibition in a randomized, double-blinded, placebo-controlled crossover trial. The short duration of the intervention allowed us to determine the weight loss-independent effects of SGLT2 inhibition on adipose tissue metabolism. We measured FFA and glucose uptake in adipose tissue by ^{11}C-palmitate and ^{18}F-fluorodeoxyglucose (FDG) PET, respectively. In addition, we measured LPL activity as well as protein and gene expression of intracellular pathways involved in adipose tissue lipid storage and lipolysis. We hypothesized that SGLT2 inhibition reduces adipose FFA uptake, thereby diverting fatty acids away from adipose tissue storage toward oxidation and ketogenesis. We also hypothesized that SGLT2 inhibition would downregulate intracellular pathways involved in lipid storage and upregulate pathways involved in lipolysis.

Research design and methods

The study was approved by the Danish Medicines Agency and The Central Denmark Region Committees on Health Research Ethics. The study was registered at eudract.ema.europa.eu (EUDRA-CT no: 2017-001779-22). Informed, written consent was obtained from all participants.

Thirteen individuals with type 2 diabetes (diabetes duration > 1 year; HbA1c 6.5–9.0% (48–75 mmol/mol) treated with metformin were randomized to empagliflozin 25 mg and placebo once-daily in a randomized, double-blinded, placebo-controlled crossover design. Patients were recruited through advertisements in local press. The study design, inclusion and exclusion criteria are described in detail in our previous publication (16). In brief, individuals with type 2 diabetes were examined after 4 weeks of empagliflozin and placebo with 1 week wash-out between the two treatment periods (Fig. 1A). By the end of each treatment period, participants were studied after an overnight fast (Fig. 1B). First, a whole-body DXA (Horizon, Hologic and Discovery, Hologic; Marlborough, MA, USA) scan was performed to assess body composition. Secondly, an adipose tissue biopsy was obtained from abdominal s.c. adipose tissue for the analysis of intracellular pathways involved in lipid storage and lipolysis. Thirdly, after 30 min of rest, patients were examined with indirect calorimetry (Jaeger Oxycon Pro, Intra medic and Deltatrac II, Datex; Gentofte, Denmark) to measure resting energy expenditure (EE) and respiratory quotient (RQ). Finally, a standard oral glucose tolerance test (OGTT) was performed with consumption of 75 g glucose. Blood was sampled at t = 0, 10, 20, 30, 60, 90, 120, 180 with measurement of glucose, insulin and free fatty acid concentrations.

Three to five days after the study day, patients were further examined with PET/CT scans (Fig. 1C). In the days between the two visits, 48 h mean glucose (Flash glucose monitoring, Flash Libre, Abbott) and 24 h blood pressure (Arteriograph 24, Tensiomed, Budapest, Hungary) were measured. Participants were also equipped with an activity tracker (Fitbit charge II, Fitbit, San Francisco, CA) and instructed to maintain an activity level equal to 6000–10,000 steps/day to ensure comparable activity levels in the two study periods.

Positron emission tomography protocols and data acquisition

^{11}C-palmitate and ^{18}F-FDG PET/CT were performed to measure adipose tissue fatty acid and glucose uptake. Due to tracer production difficulties, not all PET examinations were carried out on all participants. PET procedures, image acquisition and reconstruction parameters have been described in detail in our previous publication (16). In brief, participants were studied in the postabsorptive state

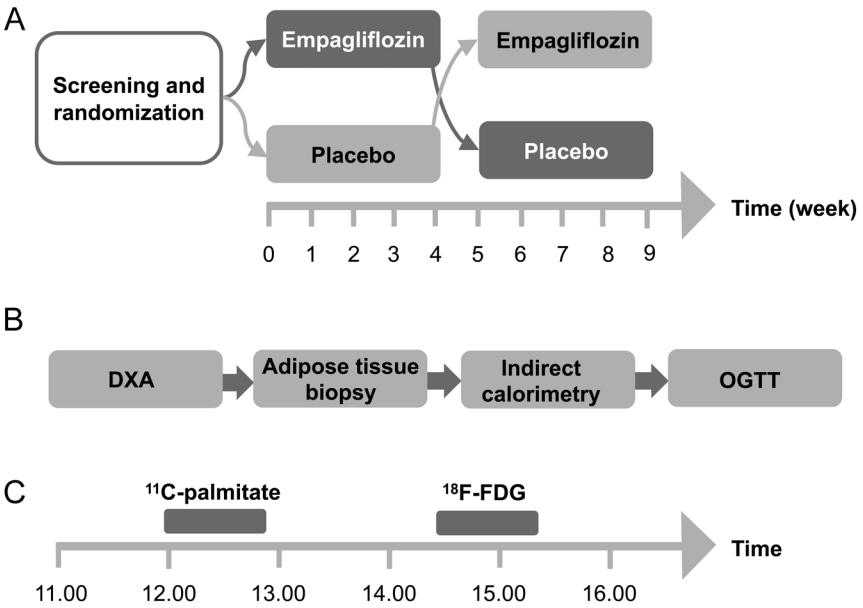

Figure 1
Study design. Patients were randomized to 4 weeks of empagliflozin and placebo in a cross-over design (A). By the end of each intervention period, participants were examined in the metabolic laboratory (B) followed by PET/CT examinations 3–5 days later (C).

after an overnight fast and took the last dose of EMPA/placebo in the morning. PET/CT examinations were done either in Siemens Biograph TruePoint TrueV 64 or Siemens Biograph Vision (Siemens Healthcare). All participants underwent PET/CT examinations on the same PET scanner on both study days. All PET scans had a field-of-view that encompassed the heart and the upper abdomen to the lower margin of the right liver lobe.

The ^{11}C-palmitate scan (n = 12) was initiated at 12:00 h with a dynamic 50-min list mode scan (frame structure 6 × 5, 6 × 10, 3 × 20, 5 × 30, 5 × 60, 8 × 150, 4 × 300 s). Data were reconstructed with a 3D iterative algorithm (3 iterations (vision 4), 21 subsets (vision 5), 5-mm Gaussian postfilter) with a voxel size of 4 × 4 × 4 mm. Blood and dynamic PET data were decay corrected to scan start time.

The 50-min dynamic ^{18}F-FDG scan (n = 10) was initiated at 14:30 h. ^{18}F-FDG of 200 MBq was injected and a 50-min list mode scan (frame structure 1 × 10, 8 × 5, 4 × 10, 3 × 20, 5 × 30, 5 × 60, 4 × 150, 4 × 300 and 1 × 600 s) was performed using 3D iterative reconstruction (3 iterations (vision 4), 21 subsets (vision 5), and 4-mm Gaussian postfilter).

PET image analysis

The input function was obtained from a VOI drawn in the aorta. To obtain tissue time–activity curves (TACs), volumes of interest (VOI's) were drawn in visceral and s.c. fat as depicted in Fig. 2. Both ^{18}F-FDG (n = 10) and ^{11}C-palmitate (n = 12) data were analyzed with a two-tissue compartment model which takes reversible radiotracer kinetics into account. The ^{11}C-palmitate uptake was calculated as previously suggested by Bucci *et al.* (17).

$$\text{FFA uptake} = \frac{C(\text{FFA}) * k1 * k3}{(k2 + k3)}$$

Whole-body palmitate clearance and lipolysis rates were estimated by:

$$\text{Clearance rate} = \frac{\text{Total tracer dose}}{\text{AUC}(\text{TAC})}$$

$$\text{Lipolysis rate} = \text{clearance} \times C(\text{FFA})$$

Blood samples

Plasma glucose concentration was analyzed immediately after sampling (YSI, Yellow Springs, Ohio, USA). Samples for the measurement of 3-hydroxybutyrate (3-OHB) were stored at –20°C and other samples were stored at –80°C until batch analysis. 3-OHB concentrations in serum were quantified using liquid chromatography-tandem mass spectrometry (18), serum FFA concentrations with an enzymatic colorimetric method assay NEFA-HR (Wako Chemicals GmbH) and insulin with AutoDELFIA immunoassay (PerkinElmer).

Adipose tissue biopsies

Adipose tissue biopsies from abdominal s.c. fat were obtained by liposuction in local anesthesia with lidocaine

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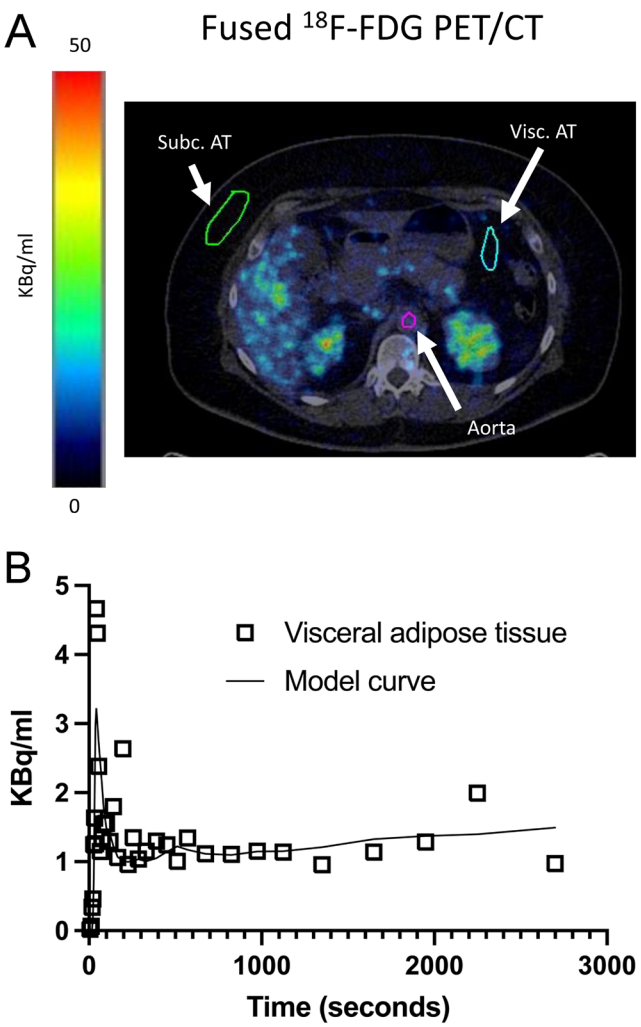

Figure 2
Drawing of VOIs on the fused PET/CT images. (A) s.c. adipose tissue VOIs were placed in the right upper abdomen, whereas the visceral adipose tissue VOIs were placed in the left upper abdomen in the area just lateral to the gastric ventricle. Care was taken to ensure the visceral VOIs did not include abdominal organs. (B) Fit of the ^{18}F-FDG 2-tissue compartmental model applied to the time–activity curves obtained from the VOIs presented in column A.

under sterile conditions. Biopsies were snap-frozen in liquid nitrogen and stored at –80°C until analysis.

LPL

Heparin-releasable lipoprotein lipase (LPL) activity was analyzed by the glycerol-stabilized method (19, 20). Lipid of 30–70 mg was defrosted and incubated in 5 U/mL heparin elution buffer (15% BSA, PBS w/ Ca and Mg; Celox Laboratories, St. Paul, MN, USA; heparin 1000 U/mL, cat. no. H-0777; Sigma) for 45 min at room temperature. The eluant was incubated in 3H-triolein-containing substrate (0.5 mCi/mL, NET-431; Perkin Elmer) for 2 h at 37°C (duplicate determinations). The reaction was stopped using methanol:chloroform:heptane (34:38:28), and after centrifugation, the supernatant was transferred to scintillation vials and counted on scintillation counter (2 × 2 determination). The interassay coefficient of variation as determined from ten repeated measurements was 11.0%. Data are expressed as µmol of FFA released per hour per gram of lipid.

Adipose tissue protein expression

Western blot analyses were performed using standard protocols and commercially available antibodies. Samples were homogenized in a buffer with pH level 7.4 containing 50 mM HEPES, 10 mM Na4P207, 20 mM NaF, 2 mM NaOV, 5 mM EDTA, 5 mM NAM, 1 mM $MgCl_2$, 137 mM NaCl, 1 mM $CaCl_2$, 2 mM DTT and HALT. Thereafter, samples were centrifuged at 13,000 rpm for 20 min. The protein containing infranatant was collected and loaded to a 4–15% Criterion TGX Stain-Free Precast Gels (Bio-Rad). Levels of GLUT4, phosphorylation of HSL and PKA phosphorylation of PLIN1 were analyzed. Phosphorylation of HSL is expressed as a ratio of total HSL level. The total protein load that was confirmed by statistical testing was similar in both groups. Detailed information regarding the antibodies is shown in the Supplementary data (see section on supplementary materials given at the end of this article).

The levels of phosphorylated AKT, PHB1, CYTC and PDH content were measured by capillary electrophoresis immunoassay (Wes, ProteinSimple, Santa Clara, CA) as previously described (21). Protein expression was quantified as peak area for the protein of interest. AKT phosphorylation is expressed as a ratio of total protein expression of AKT. The levels of PHB1, CYTC and PDH were normalized to PLIN1. Information regarding the antibodies is shown in the Supplementary data.

Adipose tissue gene expression

RNA was extracted in a mixer mill using TRIzol (Gibco BRL/Life Technologies), quantification was done using a NanoDrop 8000 Spectrophotometer (Thermo Scientific) and RNA was diluted to 50 ng/µL. The PCR reactions were performed in duplicate using 3.125 ng RNA per well in a LightCycler 480 (Roche Applied Science) using LightCycler SYBR Green master mix. mRNA levels of GLUT4, G0S2, CIDEC, PDE3B, ATGL, HSL, PLIN1, ANGPTL4, LPL,

IL6, PTEN, PGC1A and PIK3R1 were analyzed. Beta 2 microglobulin was used as the housekeeping gene and β2 microglobulin mRNA levels were similar in both interventions. All outcomes are expressed relatively to β2 microglobulin. Primers were designed using QuantPrime software (22). The primer sequences are listed in the Supplementary data.

Statistics

Data are presented as mean ± S.D. or median (95% CI) as appropriate. Data were inspected with QQ-plots for normal distribution and data were log-transformed when appropriate. Mixed model analysis was used for the analysis of data from the OGTT with treatment, time and the interaction between treatment and time as fixed factors. All other outcomes were tested with the paired samples *t*-test to detect the effects of empagliflozin compared to placebo. *P*-values less than 0.05 were considered statistically significant.

Results

Baseline characteristics, metabolic variables, body composition and energy expenditure

Thirteen metformin-treated individuals with type 2 diabetes (3 women, age: 62 ± 6 years, HbA1c: 56.7 ± 5.5 mmol/mol (7.3 ± 2.7%), diabetes duration: 4.6 ± 3.0 years, antihypertensive therapy: 62%, lipid-lowering therapy: 62%) were included in the study. As described in our previous publication (16), empagliflozin reduced 48-h mean glucose (8.0 ± 0.9 mmol/L vs 9.4 ± 2.2 mmol/L, $P < 0.01$) and insulin levels (65 ± 47 pmol/L vs 84 ± 51 pmol/L, $P = 0.01$) and increased FFA concentration (0.86 ± 0.30 mmol/L vs 0.72 ± 0.27 mmol/L, $P = 0.02$) and 3-OHB concentration (92 (95% CI: 50–169) vs 49 (95% CI: 31–79) μmol/L, $P < 0.01$) compared to placebo. Body weight (94.6 ± 9.6 kg vs 95.2 ± 9.7 kg, $P = 0.15$), total fat mass (31.4 ± 12.2 kg vs 31.2 ± 11.3 kg, $P = 0.53$) and fat percentage (32.9 ± 10.1% vs 32.4 ± 9.3%, $P = 0.26$) were similar after empagliflozin and placebo, but empagliflozin led to a small decrease in lean body mass (59.4 ± 5.6 kg vs 60.4 ± 5.4 kg, $P = 0.03$). EE was similar (7435 ± 544 kJ/day vs 7443 ± 481 kJ/day, $P = 0.95$), but RQ decreased during empagliflozin treatment (0.81 ± 0.03 vs 0.83 ± 0.03, $P = 0.02$). Since catecholamines are important regulators of lipolysis, we measured 3-methoxyadrenalin (0.20 (95% CI: 0.16–0.24) vs 0.20 (95% CI: 0.16–0.25) nmol/L, $P = 0.78$) and 3-methoxynoradrenalin (0.49 (95% CI: 0.43–0.57) vs 0.46 (95% CI: 0.39–0.55) nmol/L, $P = 0.15$) as a measure of catecholamine secretion and found no change during empagliflozin (Table 1).

Adipose tissue insulin resistance

As expected, empagliflozin reduced plasma glucose (treatment: $P < 0.0001$, time: $P < 0.0001$, interaction: $P = 0.29$) (Fig. 3A) and plasma insulin levels (treatment: $P = 0.06$, time: $P < 0.0001$, interaction: $P = 0.80$) (Fig. 3B) during OGTT. FFA were higher throughout the OGTT after empagliflozin treatment (treatment: $P < 0.001$, time: $P < 0.0001$, interaction: $P = 0.35$) (Fig. 3C). The degree of FFA suppression was not significantly affected by empagliflozin (62 ± 22% vs 69 ± 22%, $P = 0.25$). Adipose tissue insulin resistance quantified as ADIPO-IR (fasting insulin concentration × fasting FFA concentration (8) was also similar (36.7 ± 27.3 vs 37.5 ± 29.3 mmol/L × pmol/L, $P = 0.86$).

Table 1 Effect of empagliflozin on glucose, insulin, fatty acid and ketone concentration, body composition and energy expenditure.

	Empa (mean ± S.D. or median (95% CI))	**Placebo** (mean ± S.D. or median (95% CI))	***P***
48-h mean glucose, mmol/L	8.0 ± 0.9	9.4 ± 2.2	0.01
Hormone and substrate concentrations			
Insulin, pmol/L	65 ± 47	84 ± 51	0.01
FFA, mmol/L	0.86 ± 0.30	0.72 ± 0.27	0.02
Ketone bodies, μmol/L	92 (50–169)	49 (31–79)	<0.01
Body composition			
Weight, kg	94.6 ± 9.6	95.2 ± 9.7	0.15
Lean mass, kg	59.4 ± 5.6	60.3 ± 5.4	0.03
Fat mass, kg	31.4 ± 12.2	31.2 ± 11.3	0.53
Fat, %	32.9 ± 10.1	32.4 ± 9.3	0.26
Indirect calorimetry			
Energy expenditure, kJ/day	1777 ± 130	1779 ± 115	0.95
Respiratory quotient	0.81 ± 0.03	0.83 ± 0.03	0.03

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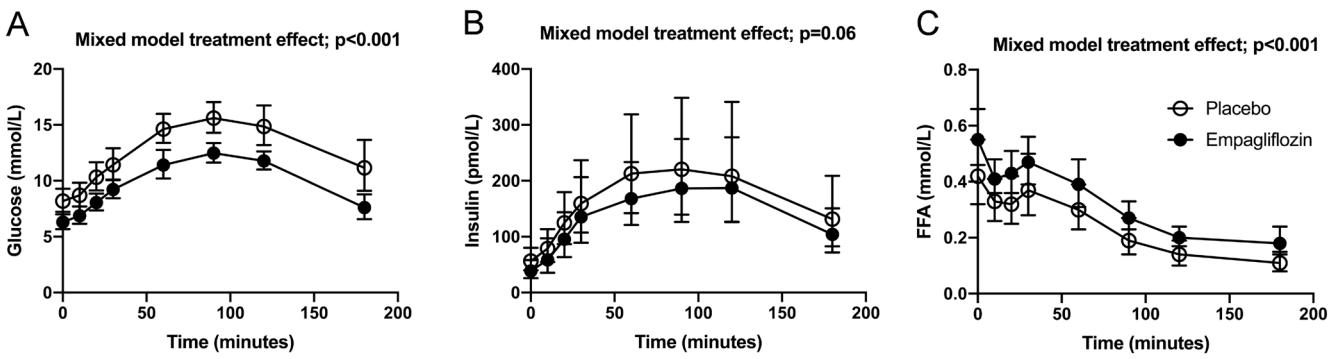

Figure 3
Glucose, insulin and FFA levels during OGTT. During OGTT, the level of glucose (A) and insulin (B) decreased, while the level of FFA was continuously elevated compared to placebo with no differences in FFA suppression fraction (P = 0.25). Data were analyzed using linear mixed model analysis. FFA, free fatty acids.

Whole-body lipolysis and clearance rate

Palmitate clearance (530 ± 94 mL/min vs 543 ± 171 mL/min, $P = 0.68$) and lipolysis rate (527 ± 249 µmol/min vs 453 ± 235 µmol/min, $P = 0.16$) were not significantly different during empagliflozin (Fig. 4A and B).

FFA and glucose uptake in adipose tissue

Empagliflozin did not significantly affect glucose uptake in abdominal adipose tissue (0.35 ± 0.36 µmol/100 g/min vs 0.77 ± 0.88 µmol/100 g/min, $P = 0.16$) or visceral adipose tissue (1.08 ± 1.03 µmol/100 g/min vs 0.99 ± 0.87 µmol/100 g/min, $P = 0.79$) (Fig. 4C and D). FFA uptake was unchanged during empagliflozin in s.c. adipose tissue (0.31 ± 0.23 µmol/100 g/min vs 0.25 ± 0.15 µmol/100 g/min, $P = 0.32$) (Fig. 4E). However, empagliflozin increased the FFA uptake in visceral adipose tissue (0.47 µmol/100 g/min (95% CI: 0.36–0.61) vs 0.37 µmol/100 g/min (95% CI: 0.26–0.52), $P < 0.05$) (Fig. 4F).

Adipose tissue protein expression

Empagliflozin reduced GLUT4 content in adipose tissue by 26% compared to placebo ($9.51 \times 10^6 \pm 4.58 \times 10^6$ vs $12.8 \times 10^6 \pm 6.46 \times 10^6$ arbitrary units (AU), $P = 0.03$) (Fig. 5A). Empagliflozin did not affect HSL phosphorylation fraction (30.9% (95% CI: 28.0–34.1) vs 33.5% (95% CI: 28.1–39.9), $P = 0.44$), PKA ($1.38 \times 10^6 \pm 1.39 \times 10^6$ vs $1.10 \times 10^6 \pm 7.25 \times 10^5$ AU, $P = 0.56$), or AKT phosphorylation fraction (11.7 ± 5.2% vs 10.4 ± 4.1%, $P = 0.18$) (Fig. 5B, C and D). Mitochondrial markers were unaffected by empagliflozin (PHB1 (1.54 ± 0.73 vs 1.69 ± 0.83, $P = 0.40$), CYTC (16.00 ± 7.95 vs 18.07 ± 9.33, $P = 0.44$) and PDH (3.08 ± 1.31 vs 3.69 ± 1.60, $P = 0.21$)).

Adipose tissue LPL activity

LPL activity was not affected by 4 weeks of empagliflozin compared to placebo (0.24 ± 0.18 vs 0.31 ± 0.15 µmol FFA/h/g tissue, $P = 0.17$) (Fig. 5E).

Adipose tissue gene expression

Empagliflozin reduced GLUT4 mRNA (0.49 (95% CI: 0.36–0.62) vs 0.66 (95% CI: 0.55–0.79) AU, $P = 0.01$) (Fig. 6A), G0S2 (71.4 ± 14.7 vs 84.4 ± 23.1 AU, $P = 0.01$) (Fig. 6B), CIDEC (0.26 ± 0.08 vs 0.30 ± 0.08 AU, $P = 0.02$) (Fig. 6C) and PDE3B (0.10 ± 0.05 vs 0.12 ± 0.05 AU, $P < 0.01$) (Fig. 6D). Gene expression of ATGL (0.28 (95% CI: 0.22–0.35) vs 0.32 (95% CI: 0.28–0.37) AU, $P = 0.20$) (Fig. 6E), HSL (1.37 ± 0.92 vs 1.58 ± 0.55 AU, $P = 0.39$ (Fig. 6F)), PLIN1 (48.1 ± 15.1 vs 53.6 ± 10.8 AU, $P = 0.09$), ANGPTL4 (1.71 ± 0.47 vs 1.65 ± 0.78 AU, $P = 0.83$), LPL (20.6 (95% CI: 15.9–26.8) vs (95% CI: 18.1 (12.9–25.4) AU, $P = 0.14$), IL6 (0.27×10^{-3} (95% CI 0.16×10^{-3}–0.46×10^{-3}) vs 0.21×10^{-3} (95% CI: 0.13×10^{-3}–0.33×10^{-3}) AU, $P = 0.50$), PTEN (0.09 (95% CI: 0.06–0.12) vs 0.10 (95% CI: 0.09–0.12) AU, $P = 0.09$), PGC1a (0.32×10^{-2} (95% CI: 0.24×10^{-2}–0.43×10^{-2}) vs 0.32×10^{-2} (95% CI: 0.24×10^{-2}–0.41×10^{-2}) AU, $P = 0.84$) and PIK3R1 (0.03 ± 0.01 vs 0.03 ± 0.01 AU, $P = 0.13$) were not affected by treatment.

Discussion

The present study was designed to provide a comprehensive characterization of the effects of SGLT2 inhibition on adipose tissue metabolism in individuals with type 2 diabetes. We applied PET/CT scans to measure adipose tissue fatty acid and glucose uptake and analyzed adipose tissue biopsies for LPL activity together with gene and

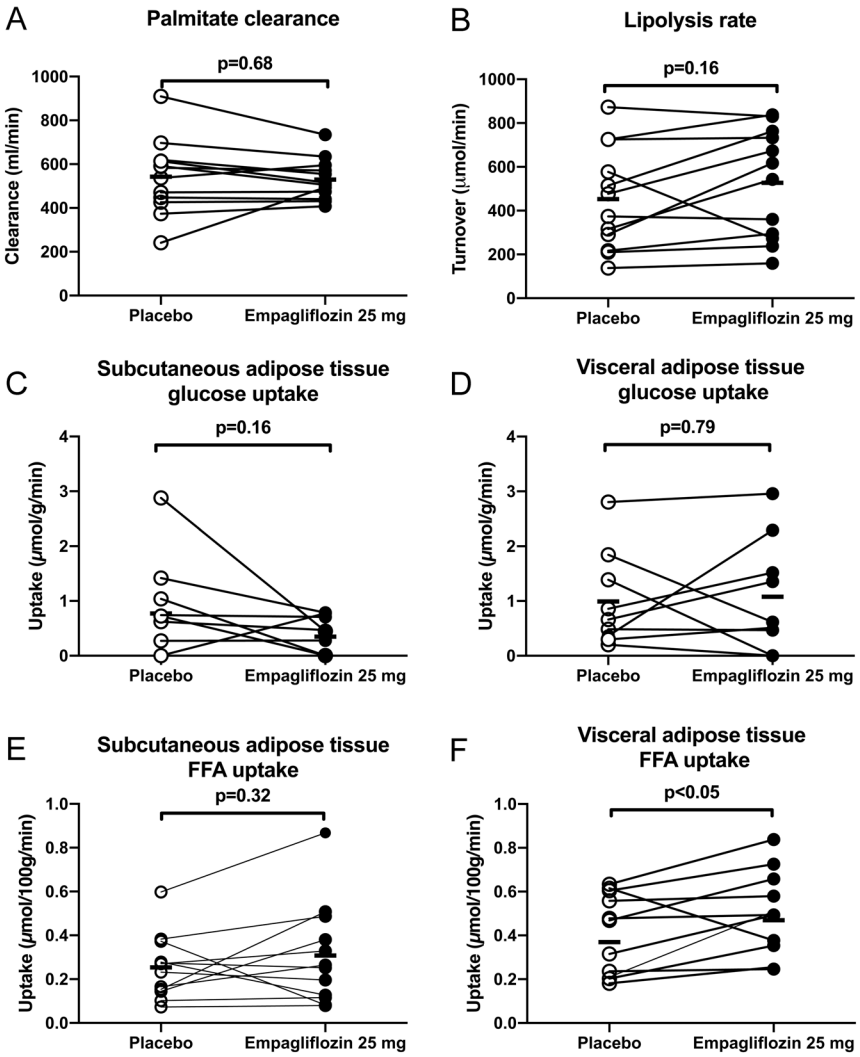

Figure 4
Whole-body palmitate clearance, lipolysis rate and adipose tissue substrate uptake. Empagliflozin did not change FFA clearance (A) or lipolysis rate (B). Empagliflozin did not affect glucose uptake in s.c. adipose tissue (C) or visceral adipose tissue (D). Empagliflozin did not affect FFA uptake in s.c. adipose tissue (E) but increased FFA uptake in visceral adipose tissue (F). Data were analyzed with a paired samples *t*-test, horizontal line = mean (A-E) or median (F).

protein expression of pathways involved in lipid storage and lipolysis. We report several novel findings. First, SGLT2 inhibition reduced gene and protein expression of GLUT4 in abdominal s.c. adipose tissue but without a detectable impact on glucose uptake. Secondly, SGLT2 inhibition did not affect fatty acid uptake in s.c. adipose tissue but increased fatty acid uptake in visceral adipose tissue. Thirdly, SGLT2 inhibition did not affect adipose tissue insulin sensitivity measured as the suppression of FFA concentration during an OGTT and ADIPO-IR but reduced the gene expression of CIDEC and PDE3B, which are involved in insulin signaling in adipose tissue.

SGLT2 inhibition reduces adipose tissue GLUT4 gene expression and protein content

GLUT4 is the most abundant glucose transporter in adipose tissue and is reduced in obesity and type 2 diabetes (23, 24). GLUT4 transports glucose into the adipocyte as a substrate for oxidation. However, glucose is also converted into glycerol which forms the backbone in triglyceride synthesis from fatty acids and by lipid storage extension. A reduction in adipose tissue GLUT4 could therefore result in release of fatty acids causing ectopic fat deposition as evidenced by pre-clinical trials. Thus, mice with adipose-selective reduction of GLUT4 are also insulin resistant in the liver and skeletal muscle (25), possibly due to increased uptake of circulating fatty acids. In the present study, 4 weeks of SGLT2 inhibition reduced the GLUT4 gene expression and protein content in adipose tissue. It is conceivable that this effect is merely secondary to reduced insulin concentrations, as demonstrated in fasting rats (26) and in rats with streptozotocin-induced diabetes (27). However, in contrast to what is observed in other metabolic disturbances associated with increased levels of circulating FFAs such as obesity and type 2 diabetes, SGLT2 inhibition

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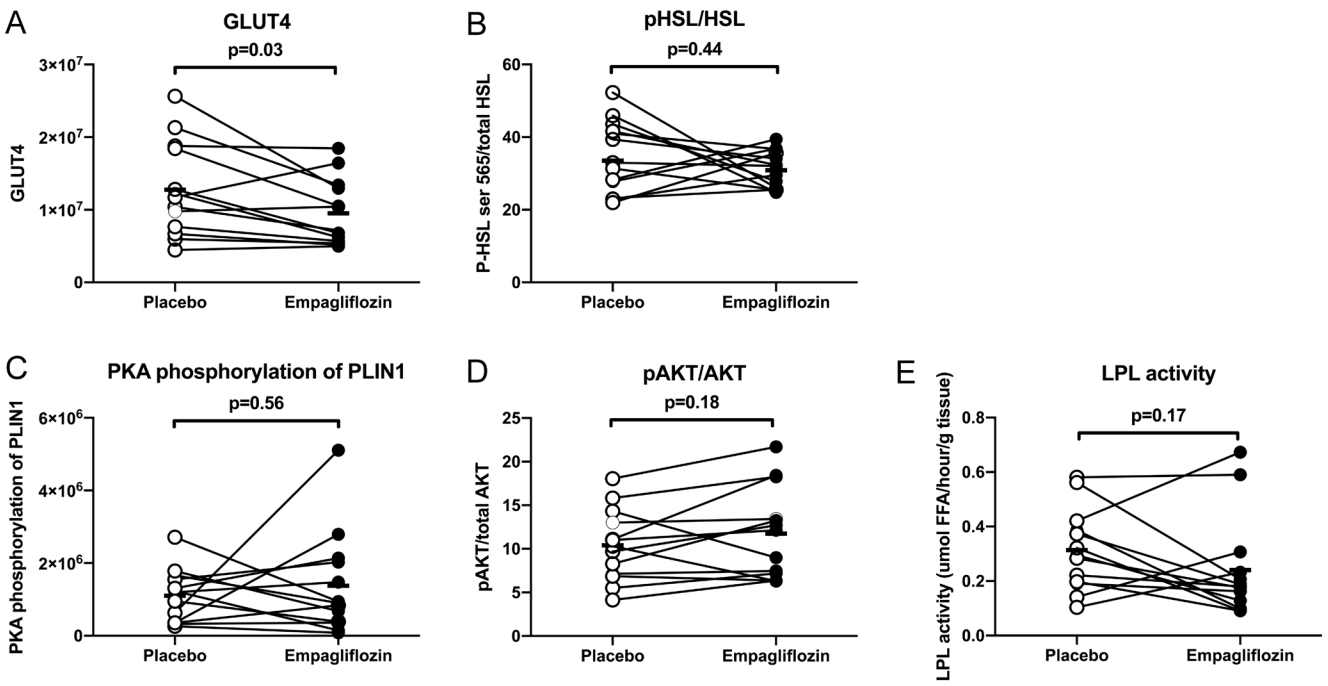

Figure 5

Protein expression of lipid storage, lipolysis and insulin signaling pathways and LPL activity in abdominal s.c. adipose tissue. Empagliflozin treatment significantly reduced protein content of GLUT4 (A) but did not affect the phosphorylation of HSL (B), PKA phosphorylation of PLIN1 (C) or phosphorylation of AKT (D) in adipose tissue. Lipoprotein lipase (LPL) enzyme activity was not affected by empagliflozin (E). Data were analyzed with a paired samples *t*-test, horizontal line = mean (A, C-E) or median (B).

increases insulin sensitivity in skeletal muscle despite reducing adipose tissue GLUT4 (28). Reduced adipose tissue GLUT4 therefore most likely does not directly cause insulin resistance in humans. Instead, the observed reduction in GLUT4 in this study probably reflects an adipose tissue substrate rebalancing away from glucose *oxidation* similar to what we recently reported for the myocardium (16). The reduction in GLUT4 gene and protein expression was not accompanied by any measurable change in adipose tissue glucose uptake. This could reflect a type 2 error due to the limited number of subjects and the low glucose uptake in adipose tissue in the postabsorptive state. In fact, adipose tissue glucose uptake was numerically 55% lower during SGLT2 inhibition, though the result was not significant ($P = 0.16$). In summary, our findings indicate that empagliflozin treatment may reduce the formation of adipose tissue triglycerides through reductions in intracellular substrate precursor availability. Such an effect of SGLT2 inhibition is supported by the observed reduction in CIDEC mRNA, which is involved in lipid droplet formation in humans (29). However, insulin-stimulated glucose uptake in adipose tissue during a hyperinsulinemic-euglycemic clamp has previously been found to be unaffected by 8 weeks of SGLT2 inhibition measured by PET/CT (30).

Therefore, SGLT2 alterations of adipose tissue glucose metabolism only appear to happen during low-insulin conditions and probably only account for a fraction of the overall metabolic changes associated with SGLT2 inhibition.

SGLT2 inhibition does not affect fatty acid uptake subcutaneous adipose tissue but increases fatty acid uptake in visceral adipose tissue

Fatty acids are primarily delivered to adipose tissue through the hydrolysis of triglyceride from circulating lipoproteins. However, FFA are also stored directly into adipose tissue (31). In the present study, we did not see any change in fatty acid uptake in abdominal s.c. tissue measured by ^{11}C-palmitate PET. Surprisingly, we did observe an increase in fatty acid uptake in visceral adipose tissue. This finding is difficult to reconcile since previous studies of SGLT2 inhibition have consistently shown reductions in visceral fat mass (32, 33). Presumably, the release of fatty acids from visceral adipose tissue is even more accelerated thereby contributing to the increase in circulating fatty acids. Since FFA concentration is linearly correlated to FFA storage (34), the increase in uptake could be explained by the SGLT2 inhibitor-driven increased delivery of FFA to visceral adipose tissue (3), but if so, a similar increase would be expected for s.c. adipose

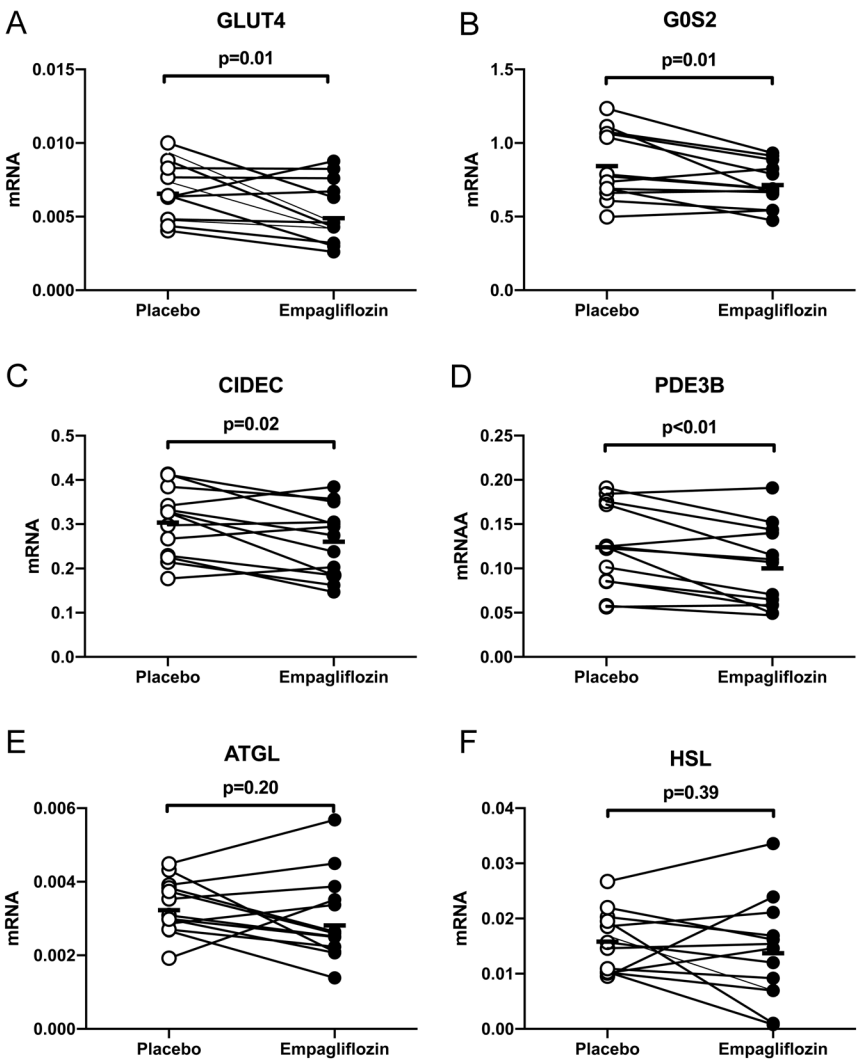

Figure 6
Gene expression of lipolysis and lipid storage pathways in abdominal s.c. adipose tissue. Empagliflozin significantly reduced the mRNA expression of GLUT4 (A), G0S2 (B), CIDEC (C) and PDE3B (D). ATGL (E) and HSL (F) were unchanged by empagliflozin treatment. Data were analyzed with a paired samples *t*-test, horizontal line = mean (B-D, F) or median (A, E).

tissue. However, visceral adipose tissue has a reduced insulin-mediated suppression of lipolysis compared to s.c. adipose tissue (35). Therefore, it could be speculated that visceral adipose tissue also has an attenuated or delayed downregulation of lipid storage secondary to the reduced insulin levels during SGLT2 inhibition compared to s.c. adipose tissue.

In short, the increased uptake probably just reflects a more rapid turnover of fatty acids from the visceral adipose tissue depot. This conclusion is inferred since the *release* of fatty acids from adipose tissue depots cannot be reliably measured by short-lived PET-tracers such as ^{11}C-palmitate.

Adipose tissue insulin sensitivity and adipose tissue insulin signaling

The regulation of lipolysis is complex and involves multiple enzymes and coregulators as recently reviewed (36). An important hallmark of type 2 diabetes is increased lipolysis and reduced adipose tissue insulin sensitivity (the ability of insulin to suppress lipolysis) (37). This may have negative metabolic consequences since an increased delivery of FFA may cause insulin resistance in skeletal muscle and the liver (38, 39). It is therefore theoretically possible that the increased lipolysis during SGLT2 inhibition could reduce skeletal muscle insulin sensitivity. However, as reported above, skeletal muscle insulin sensitivity improves rather than deteriorates during SGLT2 inhibitor treatment (28). In this study, adipose tissue insulin sensitivity was unchanged when quantified as suppression of FFA during an OGTT or as the ADIPO-IR index. The increase in circulating FFA during SGLT2 inhibition therefore most likely does not reflect an impaired adipose tissue insulin sensitivity and must be considered a less pathological state than the increased lipolysis observed in insulin-resistant states such as obesity and type 2 diabetes (40).

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Insulin signaling through AKT inhibits PKA activity with resultant inhibition of lipolytic activity. Besides a negative regulation of lipolysis, insulin signaling through AKT also stimulates lipogenesis by promoting glucose uptake and mTOR activation (41). An insulin reduction as in the present study should therefore decrease the AKT phosphorylation and increase lipolytic activity. Thus, canagliflozin treatment is associated with a ten-fold increase in HSL phosphorylation in mice (42). In this study, we observed a 15% reduction in mRNA expression of G0S2, an inhibitory protein which binds to ATGL and thereby inhibits triglyceride breakdown. This is similar to what is observed during fasting, whereas the increase in lipolysis during exercise does not affect G0S2 in humans (43). Taken together, these observations indicate that SGLT2 inhibition and fasting increase lipolysis through a similar pathway, presumably lower insulin activity (44, 45). In this study, no difference in AKT phosphorylation during SGLT2 inhibition was observed, which may be attributed to the post-absorptive and thus insulinopenic conditions of our participating subjects. Nevertheless, SGLT2 inhibition reduced both CIDEC and PDE3B mRNA, which are regulated by insulin, indicating a decrease in insulin signaling (29, 46). Ketone bodies suppress lipolysis (9), presumably through the hydroxycarboxylic acid 2 (HCA2) receptor. Therefore, the increase in circulating ketone bodies could limit the increase in lipolysis, but a small, ~ 0.1 mM, increase in our study is low compared to the affinity of beta-hydroxy-butyrate to the receptor (EC_{50} = 0.7 mM) (47). Therefore, SGLT2 inhibitor-mediated increase in ketogenesis probably has very limited effects on lipolysis. Fasting levels of metanephrines were unaffected by SGLT2 inhibitor treatment; therefore, the increase in lipolysis rate does not seem to be mediated through an increase in catecholamine levels. However, measurement of metanephrines may not exactly mirror catecholamine levels; however, the lack of increase in blood pressure and pulse rate during SGLT2 inhibition argues against an increase in catecholamine levels. Another potential regulator of lipolysis is leptin, which stimulates lipolysis in animals and cell models (48). However, leptin does only seem to have small transient effects in humans (49). Since SGLT2 inhibition decreases leptin levels (50), this should reduce lipolysis rate in contrast to the increase in lipolysis observed during SGLT2 inhibition (3).

In summary, SGLT2 inhibition does not alter adipose tissue insulin sensitivity but downregulates genes involved in insulin signaling and adipose tissue lipolysis.

Strength and limitations

The major strength of our study is the comprehensive characterization of the effects of SGLT2 inhibition on both substrate fluxes, enzyme activity, protein and gene expression in adipose tissue. However, the study also has limitations. The study was powered according to the primary endpoint which was myocardial FFA oxidation. The effects on adipose tissue were predefined secondary endpoints and the small sample size may have limited our ability to detect effects of SGLT2 inhibition; therefore, especially negative results should be interpreted with caution. Also, it should be noted that the patients were examined during postabsorptive conditions. In addition, a large number of particular gene expression variables were examined, and by extension, a number of secondary hypotheses were also tested. Although this approach may call for multiple testing corrections for example, the Bonferroni method, we felt this would be inappropriate given the exploratory nature of the study. Omitting corrections for multiple testing obviously increase the risk of type 1 statistical errors but on the other hand, reduces the risk of type 2 errors. This is likely the most appropriate in a study as small as the current. Finally, the regulation of lipid storage and lipolysis is complex and several other factors within the adipocytes may be affected by SGLT2 inhibition. In this study, we focused on factors directly involved in lipolysis and glucose and lipid transport into adipose tissue but did not measure adipokines such as leptin and adiponectin. The measurement of adipokines and other targets could have provided different results.

Conclusion

In conclusion, SGLT2 inhibition has discrete but intriguing effects on lipid storage and lipolysis in adipose tissue. We observed a reduction in GLUT4 gene and protein expression indicating a rebalancing of substrate utilization away from glucose oxidation and lipid storage capacity through reduced glycerol formation. Also, SGLT2 inhibition decreases the expression of genes involved in insulin signaling in adipose tissue. Finally, SGLT2 inhibition does not affect adipose tissue insulin sensitivity despite increased FFA concentrations.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/EC-21-0558.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Data availability
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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